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Symplastic Transport in *Ipomea tricolor* Source Leaves[1]

DEMONSTRATION OF FUNCTIONAL SYMPLASTIC CONNECTIONS FROM MESOPHYLL TO MINOR VEINS BY A NOVEL DYE-TRACER METHOD

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ABSTRACT

A novel method for the delivery of the fluorescent dye Lucifer Yellow CH to the cytosol of a source leaf mesophyll cell was devised which utilized a preencapsulation of the dye in phospholipid vesicles (liposomes). The liposomes were easily injected into the vacuoles of leaf cells of *Beta vulgaris* or *Ipomea tricolor*, where fusion with the tonoplast resulted in the release of the dye into the cytosol. Subsequent cell-to-cell movement of the dye was readily followed by fluorescence microscopy. Using this liposome technique symplastic continuity from the the mesophyll to the minor veins of the source leaf of *Ipomea tricolor* was demonstrated. This agreed with ultrastructural studies which demonstrated the presence of plasmodesmata between all cells from the mesophyll to the minor veins. The symplastic movement of dye from the injected mesophyll cell to the minor veins was unaffected by pretreatment of the leaf tissues with 2 millimolar *p*-chloromercuribenzenesulfonic acid. Pretreatment of the leaf tissues at alkaline pH (3-[*N*-morpholino] propanesulfonic acid-KOH, pH 8.0) had no apparent effect on dye movement between adjacent mesophyll cells but inhibited the movement of dye into and along the minor veins. Thus, although there were no apparent barriers to symplastic solute movement in this leaf, symplastic barriers could be imposed by the experimental conditions used.

The transport of photosynthetically derived solutes from sites of synthesis in the source leaf mesophyll to the phloem of the minor veins has been under intensive investigation in recent years. At present, solutes are thought to move through the mesophyll symplastically, from cell to cell through plasmodesmata, but are then thought to exit into the apoplast at some point close to the sieve element/companion cell complex followed by active uptake into the phloem. This active uptake step establishes the concentration gradient that has been demonstrated to exist between the sieve element/companion cell complex and adjacent cells. The data supporting this putative apoplastic step in phloem loading have been extensively reviewed (9, 11) but are still open to question (17). The study of apoplastic transport in plant tissues therefore continues to receive considerable attention from many researchers interested in phloem transport.

In contrast, the study of symplastic transport has been relatively neglected by most investigators. This is largely due to the paucity of experimental techniques suitable for investigation of solute movement in the symplast. Most studies have been limited predominantly to the measurement of the frequency and distribution of plasmodesmata along the route from the mesophyll to the minor veins (*e.g.* 8, 15, 27). These measurements, coupled with measured rates of assimilate flux between the sites of synthesis and transport, have been used as qualitative evidence for the feasibility of symplastic transport (15, 22) but cannot prove the functionality of such a route *in vivo*.

The adaptation of neurological dye tracer techniques for use in plants has now provided a means for establishing the extent of the functional symplast in plant tissues (4–6, 12, 13, 30). A major drawback to the use of these techniques in vacuolate tissues, such as mature source leaves, is the number of injections that occur into the vacuole. Dye injected directly into the vacuole often fails to move symplastically to adjacent cells. The research reported here describes a novel method for the delivery of a fluorescent tracer to the cytosol of a source leaf mesophyll cell. Our approach involves the preencapsulation of the dye in phospholipid vesicles (liposomes) and actually exploits the ease by which vacuolar injections can be made.

We have mapped the symplastic connections in the source leaf of *Ipomea tricolor* using this liposome injection technique to introduce the fluorescent dye, Lucifer Yellow CH. In agreement with a recent report on symplastic continuity in source leaves of *Commelina cyanea* (6), we have found no apparent barrier to symplastic solute movement along the entire pathway from the mesophyll to the minor veins. More importantly, we have found that barriers to symplastic transport can be induced by certain treatments commonly used to manipulate the apoplastic uptake of sugars into leaf tissues.

MATERIALS AND METHODS

Plant Material. Fully expanded source leaves from 4-week old *Ipomea tricolor* cv "Heavenly Blue" (Burpee Seeds) or 5-week old *Beta vulgaris* cv SSNB1 were used for all experiments. Plants were germinated and grown in a controlled environment chamber under conditions described previously (14).

Chemicals. Phosphatidylserine (beef brain), phosphatidylcholine (egg yolk), cholesterol, stearylamine, PCMBS,[2] Mes, Mops, and Lucifer Yellow CH were purchased from Sigma Chemicals. Fluorotrichloromethane (Freon 11) was obtained from Aldrich Chemicals.

Liposome Preparation. Liposomes were prepared by the freeze-thaw method of Pick (25). All procedures were carried out under a N_2 atmosphere to avoid oxidation of phospholipids. Twenty mg of phospholipid (either pure phosphatidylserine or a mixture of phosphatidylcholine:stearylamine:cholesterol in a molar ratio of 5:1:5) in chloroform was placed on ice in a 12 × 35 mm shell

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[2] Abbreviations: PCMBS, *p*-chloromercuribenzenesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid.

vial and taken to dryness under a stream of N_2 gas. To allow complete transferral of the resulting phospholipid film from the sides of the vial, the phospholipid was first redissolved in 0.5 ml ice-cold Freon 11 (29). An ice-cold solution (0.5ml, 20 mg ml^{-1}) of Lucifer Yellow CH was then added, and the phospholipid/dye mixture was vigorously stirred on a vortex mixer until it had reached room temperature and the Freon (b.p. 23.5°C) had boiled off. Final traces of Freon were removed by briefly placing the vial in a 300°C water bath.

Small unilamellar liposomes were generated by sonication of the phospholipid suspension for 2 min using a Branson 200 Sonifier Cell Disrupter equipped with a 3.17 mM microprobe. The preparation was frozen in liquid N_2 and thawed at room temperature to increase the size and trapping efficiency of the liposomes (25). After a brief 30 s resonication and filtration through a 0.2 μm membrane filter, the liposomes were separated from unencapsulated dye by gel filtration through 2 ml columns of Sephadex G-50 equilibrated with 20 mM KCl. Liposomes eluted in the void volume, which was monitored by Blue Dextran elution through similar gel columns.

Microinjection Procedures. Liposomes were back-loaded by capillary action into the tips of glass micropipettes of 0.5 to 1.0 μm tip diameter. The micropipettes were sealed into a micropipette holder equipped with a Luer port (model MPH-1, WPI Instruments, New Haven, CT) which was connected by a length of plastic tubing to a 1 ml gas-tight Hamilton syringe. The holder, tubing, and syringe were filled with water to provide a hydraulic system for microinjection of the liposomes. The injection pressure was controlled by a screw-driven attachment which regulated depression of the syringe plunger. The approach and impalement by the micropipette was controlled by an hydraulically driven micromanipulator (model MO-102, Narishige Scientific Instrument Laboratory, Tokyo, Japan) and cell impaler (Narishige model MM-333).

A mature source leaf, still attached to the intact plant, was fastened abaxial side up to a microscope slide with double-sided transparent tape and positioned on the microscope stage. The leaf was equilibrated for a 1 h period under a fiber-optic light source (Schott model KL1500) which provided a light intensity of 200 μmol photons m^{-2} s^{-1} (PAR, 400–700 nm) at the level of the microscope stage and 50 to 100 μmol photons m^{-2} s^{-1} over the rest of the plant. A small well of modeling clay positioned on the leaf surface was filled with a bathing medium consisting of 20 mM Mes-KOH (pH 5.0), or with distilled H_2O. A 5 mm^2 area of the leaf epidermis was peeled back to expose the injection site. The spongy mesophyll cell to be injected was localized with substage bright field illumination using a 25× long working distance objective and carefully impaled with the micropipette and injected with the liposome suspension. Liposome fusion and movement of the dye was observed by epifluorescence microscopy using a Leitz Orthoplan microscope equipped with a violet (BP 436/437) excitation filter, an RKP 475 mirror, and an LP 490 barrier filter. An additional 640 nm cut-off filter (Ditric Optics Inc., Hudson, MA) was placed between the microscope and camera to suppress Chl fluorescence. Photographs were taken on Kodak Tri-X Pan film exposed at 1000 ASA and push-processed using Acufine developer (Acufine, Chicago).

PCMBS and pH Treatments. For some experiments the bathing medium irrigating the injection site was changed to allow pretreatment of the leaf tissue with PCMBS or alkaline pH. For these experiments, two injection sites were prepared on each leaf to allow control and treatment experiments to be run concurrently on the same leaf tissue.

For the PCMBS pretreatment, the protocol of Giaquinta (10) was followed, and the bathing media consisted of 20 mM K-phosphate (pH 7.0) with or without 2 mM PCMBS. Both injection sites were initially pretreated for 45 min with K-phosphate (pH 7.0). The buffer was then removed and replaced with fresh buffer (control) or buffer plus 2 mM PCMBS (experimental). After 15 min the solutions in both wells were replaced with fresh buffer containing no PCMBS and injections were performed as described above. Dye movement was followed for a 1 h period after the injections.

Because the use of phosphate buffer was found to be deleterious to the movement of Lucifer Yellow in the source leaf, experiments were also run in which peeled areas were first pretreated with 2 mM PCMBS dissolved in water, followed by injection in Mes buffer as described above.

For the pH treatment, the protocol of Turgeon (31) was followed, and the bathing media consisted of 25 mM Mes-KOH plus 20 mM $CaCl_2$ (pH 5.5, control) or 25 mM Mops-KOH plus 20 mM $CaCl_2$ (pH 8.0, experimental). The injection sites were pretreated for 1 h at the selected pH, fresh buffer of the appropriate pH was placed in each well, and the injections were performed as described above. Again, dye movement was followed for a 1 h period.

Leaf Structural and Ultrastructural Studies. To assess the ultrastructural status of the injected leaf material, tissue preparations were made from both liposome-injected (experimental) and noninjected (control) regions of source leaves of *Ipomea*. Relatively large segments (up to 1.0 cm^2) of experimental and control tissues were excised and transferred to vials containing ice-cold fixative consisting of 1.6% (w/v) depolymerized paraformaldehyde, 2% (w/v) glutaraldehyde, 0.5% (w/v) tannic acid, and 2 mM $CaCl_2$ in 50 mM Na-cacodylate buffer (final pH 7.0). After 1.5 h, the leaf segments were cut into smaller pieces (approximately 20 mm^2) and allowed to fix for an additional 4 h at 15°C. The segments were postfixed overnight at 4°C in 1% (w/v) OsO_4 in 50 mM Na-cacodylate buffer (pH 7.0), then dehydrated in a graded acetone series and embedded in Spurr's resin (28). Sections were cut using conventional methods, mounted on formvar-coated slot grids and stained with aqueous uranyl acetate followed by Reynold's lead citrate (26). Sections were viewed and photographed with a JEOL 100S electron microscope operated at 80 keV.

Light micrographs of fixed tissues were obtained from semithin sections (approximately 0.75 μm) cut from plastic-embedded leaf segments prepared for EM as above. The sections were cut with glass knives and stained with 0.065% (w/v) toluidine blue in 1% (w/v) borate buffer. Sections were viewed and photographed using a Leitz Orthoplan microscope.

Cryosectioning Techniques. To determine the cellular location of the Lucifer Yellow following its movement in the source leaf, a section of the leaf corresponding to the injection site and the surrounding area through which the Lucifer Yellow had been seen to move was excised and thinly coated with Tissue-Tek O.C.T. compound (VWR Scientific). To allow cross-sections to be made, the tissue was inserted perpendicularly into an agar block fastened to the surface of a cryotome chuck, and then sealed in place with Tissue-Tek. The entire cryotome chuck was then dropped into a slot in a brass block held at liquid N_2 temperature. Following freezing, the cryotome chuck was sealed in a cylinder of aluminum foil, also held at liquid N_2 temperature, and then placed in a glass scintillation vial held in dry ice. The entire freezing procedure was completed within 1 min from the time of excision of the injected tissue from the intact leaf. The tissue was stored in the sealed vial in a −70°C freezer for 2 d prior to cryosectioning.

Cross-sections of 10 μm thickness were cut from the frozen leaf tissues, at −25°C using a cryostat (Damon/IEC model 3398), then mounted on formvar-coated slides, and stored for 2 d at −45°C over Drierite. The sections were then slowly warmed to room temperature, mounted in immersion oil, and viewed by

differential interference contrast and fluorescence optics with a Leitz Orthoplan microscope.

RESULTS

Liposome Fusion and Movement of Lucifer Yellow CH. The effects of phospholipid composition of the microinjected liposomes on the subsequent delivery of Lucifer Yellow CH to the cytosol of source leaf mesophyll cells of *Beta vulgaris* is illustrated in Figure 1. Positively charged liposomes composed of a molar ratio of 5:5:1 phosphatidylcholine:cholesterol:stearylamine, which have been used successfully for delivery of solutes to plant cell protoplasts (2), were ineffective in delivering Lucifer Yellow to the cytosol when microinjected into the vacuole. No dye movement was seen out of the injected cell (Fig. 1a), even after 15 min following the injection (Fig. 1b). The dye instead appeared to remain inside the vacuole, indicating that liposomes of this composition were not fusing with the tonoplast. In contrast, negatively charged liposomes prepared from pure phosphatidylserine appeared to fuse readily with the tonoplast, as evidenced by the rapid delivery of the dye to the cytosol (Fig. 1, c, e, f, and g). The particular efficacy of negatively charged phosphatidylserine liposomes in the delivery of Lucifer Yellow to the cytosol is, we suspect, most probably a result of a high degree of fusion of this phospholipid under vacuolar conditions. Fusion of phosphatidylserine vesicles is known to be particularly sensitive to Ca^{2+} ions, especially at a low pH (24).

Extensive movement of the dye from the injected cell to adjacent cells was recorded. In Figure 1c it can be seen that the dye had spread in a lateral direction, within 30 min, to mesophyll cells 10 cells away from the injection site (*i.e.* a distance of approximately 300 μm). Movement to cells underlying the injection site also occurred (Fig. 1e, f, and g),l but the extent to which dye movement could be visualized by light microscopy was limited, due to the compactness and thickness of the spongy mesophyll layers of the *Beta* leaf (Fig. 1d). Consequently, we could not detect movement of Lucifer Yellow into the minor veins of sugar beet source leaf tissue.

Dye Movement in *Ipomea tricolor* Source Leaves. Source leaves of *Ipomea tricolor* possess a much less dense mesophyll (Fig. 2 a, d and e); consequently, the minor veins were readily visible with the light microscope. In addition, the lower epidermis was easily removed, with minor damage being inflicted on the neighboring cells (Fig. 2 d and e). (During the course of these experiments, it was also found that prolonged illumination of the tissues with the epifluorescence light source could cause a further degree of tissue disruption. In this case, the damage was minimized by exposing the leaf to the light source only during the photographic exposures.) Removal of the epidermis allowed us to gain access to unperturbed spongy mesophyll cells in close proximity to minor veins (Fig. 2 a and c). Furthermore, in this leaf the space between the sieve elements and the epidermis is occupied by few cells (Fig. 2 a and b). Thus, if Lucifer Yellow were to move into the minor veins (sieve elements?), this anatomy would provide an optimal setting for its detection.

Microinjection of phosphatidylserine-encapsulated Lucifer Yellow into spongy mesophyll cells of *I. tricolor* also resulted in rapid dye release to the cytosol. Dye was evident in mesophyll cells adjacent to the injected cell 6 min after injection (Fig. 3, a and b). By 16 min, we could detect the movement of the dye into and along the minor veins of the leaf (Fig. 3, c and d). By 30 min, minor veins 1 mm away from the injection site showed considerable fluorescence, indicating a very extensive movement of dye in the symplast of this leaf (Fig. 3, e and f). Cryosections of the regions of the vasculature through which the Lucifer Yellow had moved showed that the fluorescence was confined to the bundle sheath and regions of the phloem tissue (Fig. 4). However, the specific phloem cell(s) which contained the Lucifer Yellow could not be discerned by this method.

Symplastic Continuity in *Ipomea* Leaves. An electron microscopic study on the mature source leaf of *I. tricolor* was conducted to ascertain the extent of symplastic continuity in this particular tissue. In addition, we sought to establish the effect(s), if any, that Lucifer Yellow, per se, might have had on the plasmodesmata.

The presence of plasmodesmata in the common walls between (a) mesophyll cells (Fig. 5, a and b), (b) mesophyll and bundle sheath cells (Fig. 5d), (c) bundle sheath and companion cells (Fig. 5f), and (d) companion cells and sieve elements (Fig. 5h). indicated symplastic continuity between all cells from the mesophyll to the sieve elements. Our fixation protocol enabled us to determine the fine structure of the various plasmodesmatal types in the *Ipomea* leaf. There were certain subtle structural differences in the plasmodesmata interconnecting mesophyll, bundle sheath, phloem parenchyma, and companion cells, but in general, the plasmodesmata shared a number of common features. Extensive median cavities, with multiple extensions to the protoplasts on either side of the wall, were frequently observed (Fig. 5, a and f). The extensions were associated with neck constrictions that occluded the cytoplasmic annulus of the plasmodesma (Fig. 5b). The axial components of these plasmodesmata were often swollen in the mid-region of the wall (Fig. 5d). ER was frequently associated with the cytoplasmic ends of the plasmodesmata (Fig. 5b). Branched plasmodesmata typical of those described for most dicotyledonous species (7) were found between companion cells and sieve elements (Fig. 5h).

Of more importance to the present study was the demonstration that movement of Lucifer Yellow through the leaf tissue had no detectable effect on plasmodesmatal ultrastructure. This is clearly shown in Figure 5 (*cf.* a, b, d, f, and h, with c, e, g, and i).

Effects of PCMBS and Alkaline pH Pretreatments on Dye Movement. Studies on many different leaf types have shown that uptake of exogenously supplied sugars into leaf tissues is reduced by pretreatment of the tissues with the sulfhydryl group blocking agent PCMBS or by pretreatment at alkaline pH (10, 11, 31). The effect of either of these two treatments on symplastic transport in source leaf tissues had not yet been addressed and was investigated here.

The sulfhydryl group inhibitor PCMBS had no apparent effect on the movement of Lucifer yellow in the symplast of *Ipomea* source leaves (Fig. 6). Although the movement along the veins was less extensive than that seen previously after a 30 min postinjection period (*cf.* Figs. 3e and 6, a and c), this appeared to be due to the phosphate buffer (pH 7.0) used in these experiments as dye movement was equally restricted in both the control (Fig. 6, a and b) and the PCMBS-treated (Fig. 6, c and d) tissues. Extensive dye movement following PCMBS treatment was observed if phosphate buffer was eliminated and the tissues were instead pretreated with an aqueous solution of PCMBS and then bathed in a Mes-KOH buffer at pH 5.0 for the injection and postinjection periods (Fig. 6, e and f).

In contrast to the results obtained with PCMBS, treatment of leaf tissues at an alkaline pH had a dramatic effect on the movement of Lucifer Yellow in the symplast of the *Ipomea* leaf (Fig. 7). At pH 5.5, the usual dye distribution pattern was seen between adjacent cells and then into and along the minor veins (Fig. 7, a and b). At pH 8.0, the dye still moved extensively but often only between mesophyll cells (Fig. 7, c and d). In 7 out of 11 experiments, no movement into the minor veins took place at this pH.

DISCUSSION

Convincing evidence for a unified, operative symplastic network has now been presented for a number of plant tissues,

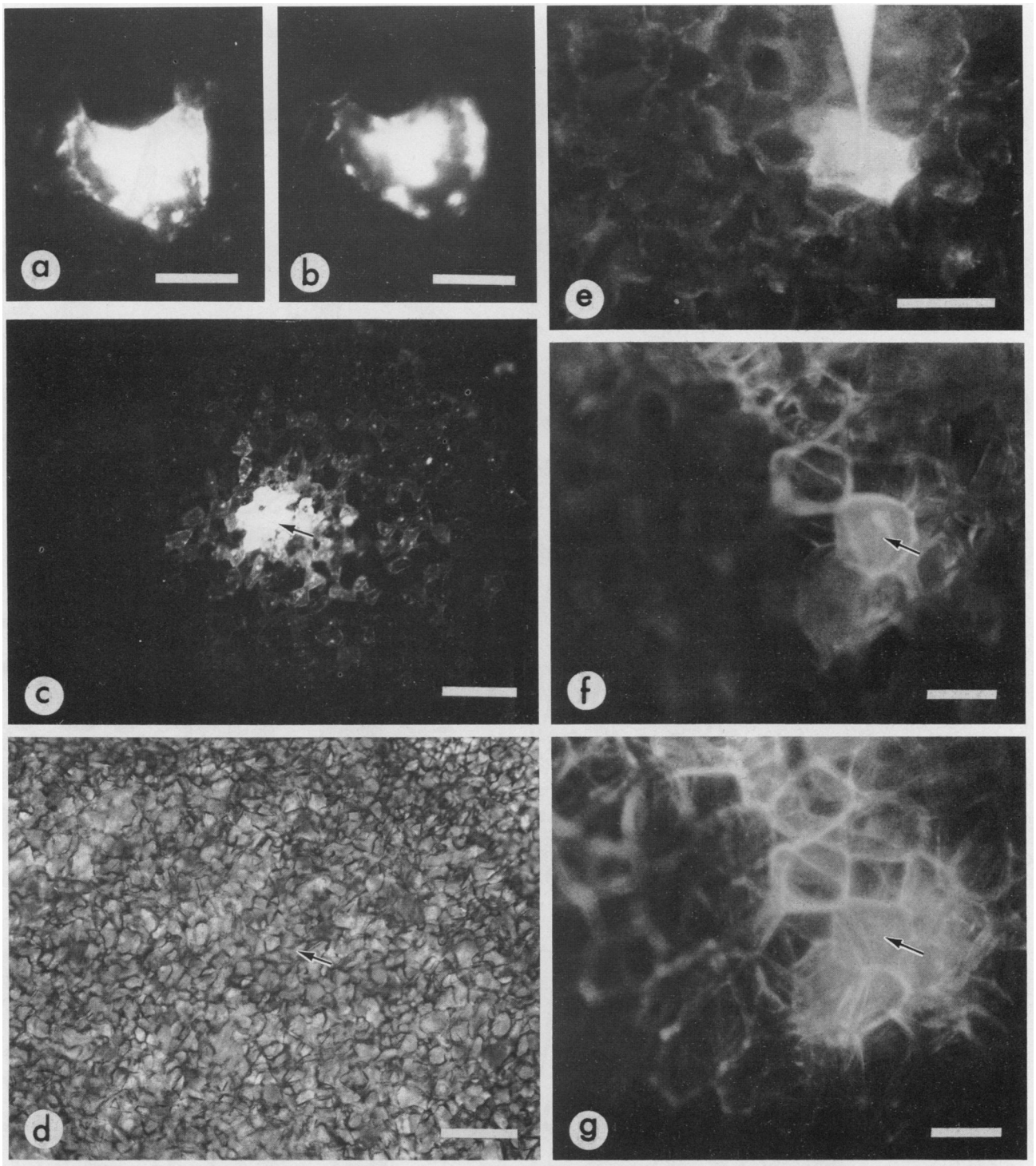

FIG. 1. Movement of Lucifer Yellow CH in the source leaf symplast of *B. vulgaris* following microinjection of liposome-encapsulated dye into a spongy mesophyll cell. Injected cells are indicated by arrows. All bars = 25 μm. Nonmovement of dye 5 min (a) and 15 min (b) after injection of Lucifer Yellow encapsulated in positively charged liposomes (phosphatidylcholine:stearylamine:cholesterol; 5:1:5 μmol). Movement of dye following injection of Lucifer Yellow encapsulated in negatively charged liposomes (phosphatidylserine): (c), 30 min after injection (×64); (d), bright field micrograph of injected area depicted in (c); (e), leaf cell during injection; (f) and (g), dye movement after 5 and 15 min, respectively.

including the leaf of *Elodea* (4), leaf and extrastelar tissues of *Egeria* (3, 5, 12) apices of *Silene* (13), leaf tissues of *Allium* (23), staminal hairs of *Setcreasea* (30), and the source leaf of *Commelina* (6). Movement of tracer dyes has been shown to be controlled by changes in cellular concentrations of ions, such as Ca^{2+} and aromatic amino acids (3, 4). In addition, size exclusion limits have been established for symplastic movement in some tissues (5, 12, 13, 30) which can apparently be overcome by treatments, such as plasmolysis (4). In other studies, barriers to symplastic movement have been found even in regions where apparently normal plasmodesmata are present, suggesting the existence of symplastic "domains" within specific plant tissues (5).

The similarity of our results to those reported for symplastic movement of Lucifer Yellow in source leaves of *Commelina* (6), is convincing evidence that liposome encapsulation is a valid

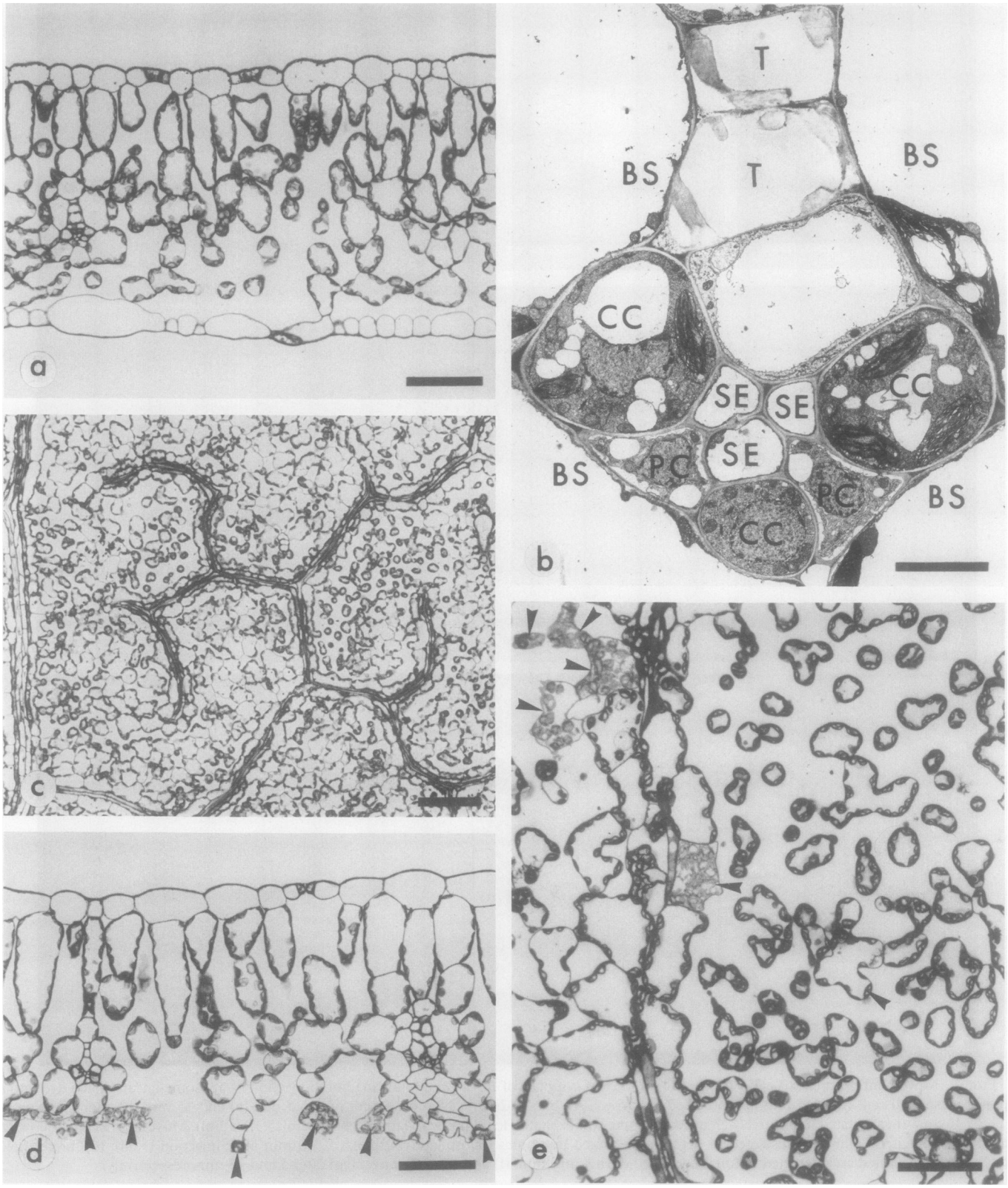

FIG. 2. Light and electron micrographs of *I. tricolor* source leaves showing the anatomical organization of the tissue used for dye tracer studies. (a) Leaf cross section. Bar = 50 μm (×270). (b), Cross-section of a minor vein and portions of the surrounding bundle sheath. Companion (CC) and phloem parenchyma cells (PC) are in contact with the bundle sheath cells (BS). (SE: sieve element, T: tracheary element). Bar = 5μm (×3300). (c), Paradermal section taken at the level of the phloem demonstrating the typical vascular pattern and the extent of lateral interconnections between the mesophyll cells at this level. Bar = 100 μm (×110). (d), Cross-section of an area of leaf injected with liposome-encapsulated Lucifer Yellow CH. Darts indicate cells damaged by removal of the lower epidermis and/or epifluorescence illumination. Bar = 5 μm (×275). (e), Paradermal section taken at the lowest level of the phloem in the injected tissue. Darts indicate cells damaged by removal of the lower epidermis and/or epifluorescence illumination. Bar = 50 μm (×300).

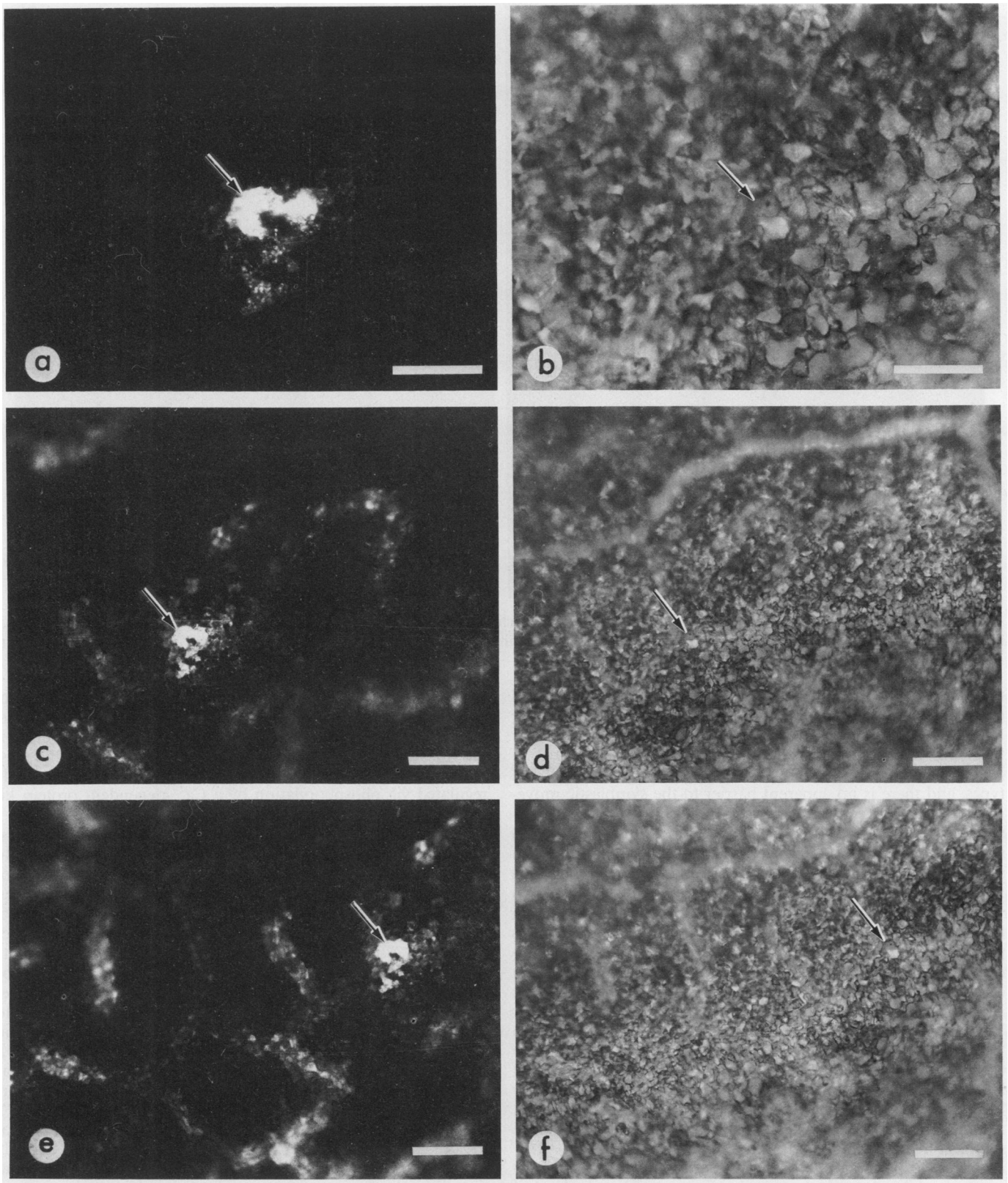

FIG. 3. Movement of Lucifer Yellow in the source leaf symplast of *I. tricolor* following microinjection of liposome-encapsulated dye into a spongy mesophyll cell. Arrows indicate the site of injection. Extent of dye movement 6 min (a), 16 min (c), and 30 min (e) after injection. Bright field micrographs (b), (d), and (f) depict regions illustrated in (a), (c), and (e), respectively. (a) and (b), Bar = 100 μm (×160); (c–f), bar = 200 μm (×64).

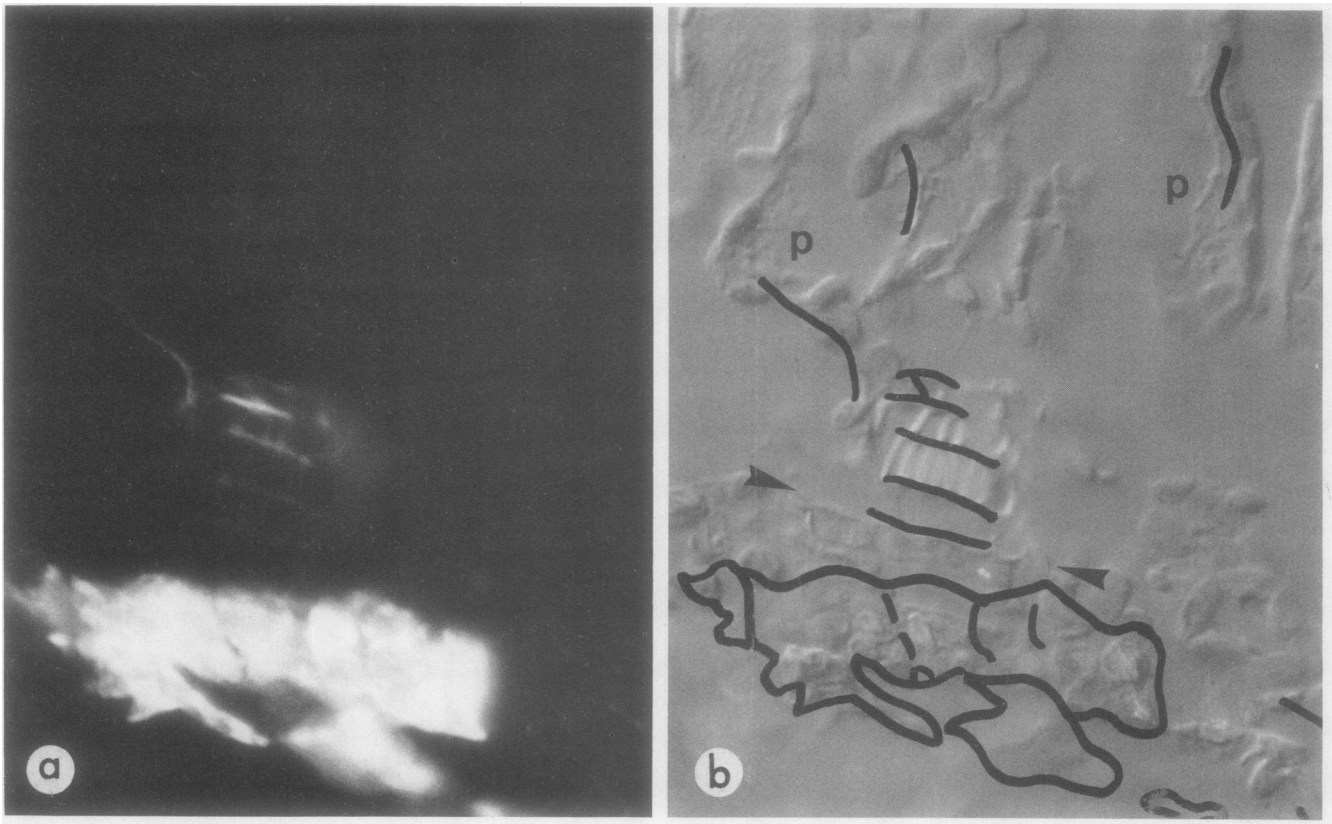

FIG. 4. Cryosection of vascular tissue of an *Ipomea* leaf through which Lucifer Yellow has moved. (a), Fluorescence micrograph; (b), differential interference contrast micrograph of the same section. Area delineated in (b) corresponds to fluorescent area depicted in (a). Darts in (b) indicate interface between xylem and phloem tissues. p, Palisade cell.

technique for the delivery of intercellular probes to the cytosol of vacuolate cells. The data presented here indicate that there are fully functional symplastic connections along the entire pathway from the mesophyll to the minor veins in the source leaf of *Ipomea*. Plasmodesmata can be found interconnecting all cell types, and there is no apparent barrier to the symplastic movement of Lucifer Yellow CH between these cells. The free permeability of the relatively small Lucifer Yellow molecule (457 D) suggests that there is no permeability barrier to the diffusion of small molecules throughout the leaf symplast. However, barriers to symplastic transport can be artifically imposed.

The image obtained of the distribution of Lucifer Yellow in the *Ipomea* leaf symplast following alkaline pH treatment is particularly interesting, especially when compared to autoradiographic images which have been reported for the distribution of exogenous [^{14}C]sucrose in leaf discs pretreated at high pH (31). A preferential localization of 'label', either [^{14}C]sucrose or Lucifer Yellow, is seen in the leaf mesophyll at high pH with little or no 'activity' in the minor veins. The effect on pH on sugar uptake is usually attributed to a reduction in sucrose-proton co-transport by the minor veins at high pH (10, 11). The data shown here for Lucifer Yellow suggest an alternative possibility; namely, that alkaline pH treatment disrupts the relay of solutes through the symplast from the mesophyll to the minor veins.

Current experimental evidence suggests that the free movement of solutes through the symplast is regulated at the level of the plasmodesmata (3–5). The actual mechanisms controlling movement through plasmodesmata are unknown at present, but modulation of intracellular Ca levels is implicated (3). The action of Ca is apparently not through its effects on cytoplasmic streaming or on callose deposition (5). Electron-dense granules with Ca^{2+}-ATPase activity appear at the neck region of the plasmodesma in response to Ca^{2+} treatment (1). This observation has been used to implicate ATPase activity, associated with plasmodesmata, in the control of solute movement through the symplast (5), but direct experimental evidence is lacking. However, it is interesting to speculate on such a role, particularly as the plasmalemma H^+-ATPase is one protein thought to be metabolically regulated via phosphorylation by a Ca^{2+}-dependent protein kinase (33).

Regulation of symplastic solute flow at the level of the plasmodesmatal ATPase could explain the nonmovement of dye to the veins at high pH (Fig. 6). It may also explain the lack of effect of PCMBS treatment on movement of Lucifer Yellow seen in this study (Fig. 5), if, as current data appear to indicate (16, 21), the action of PCMBS is predominantly on the sucrose carrier and not on ATPase activity. The data presented here also indicate that plasmodesmata in different regions of the leaf may be regulated independently. Subsequent carbon partitioning between phloem-mobile and nontransported compounds would also be affected, as indicated in previous biochemical studies on stachyose synthesis in *Cucurbita* in response to plasmolysis (19).

The involvement of apoplastic or symplastic routes for sugar movement in source leaves appears to be much less straightforward than first envisioned in early phloem transport work (17). Most evidence now indicates that a considerable proportion of the uptake of sugar once attributed to 'phloem loading' actually represents uptake into nonphloem tissues (17, 18, 20, 32) and that the contribution of the vasculature to this uptake may, in fact, be small (32).

The data in the present study further illustrate the complexities associated with studying phloem loading. Clearly, studies based solely on structural detail are of only limited value in predicting the efficacy of the symplastic pathway in any source leaf tissue. It is essential that we now develop a better understanding of plasmodesmatal function and integrate this knowledge into stud-

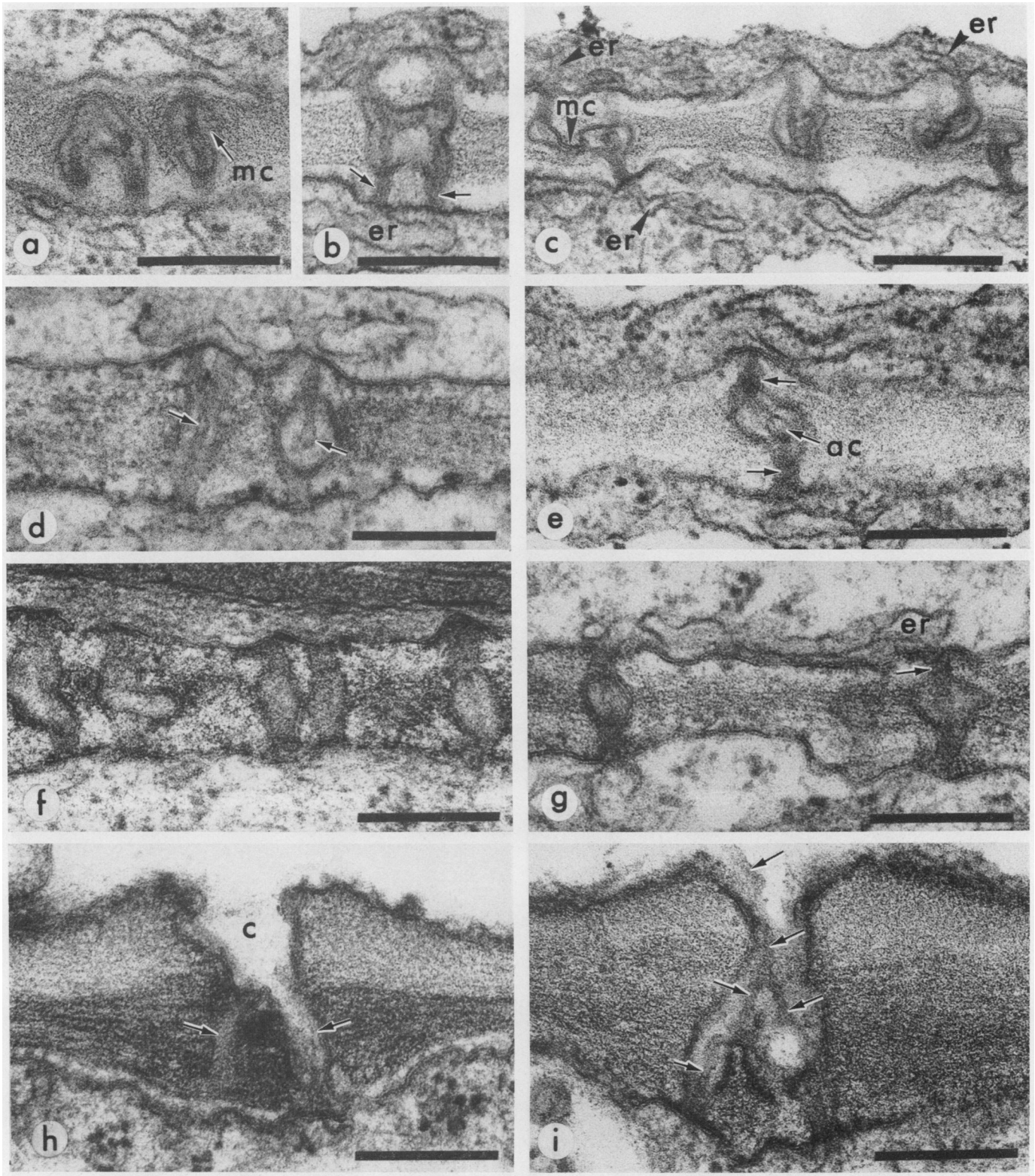

FIG. 5. Structure of plasmodesmata occurring along the pathway from mesophyll cells to the sieve elements of minor veins in the source leaf of *I. tricolor*. Representative plasmodesmata from control tissues (a, b, d, f, and h) and liposome-injected tissues through which Lucifer Yellow has moved (c, e, g, and i) are shown. All bars = 0.25 μm. (ac), Axial component (desmotubule); (er), endoplasmic reticulum; (mc), median cavity. Plasmodesmata interconnecting mesophyll cells from control (a, b) and injected (c) tissues. Arrows in (b) indicate neck constrictions; note apparent continuity between er and neck constrictions. Darts in (c) indicate horizontally oriented mc and three sites of possible contact between the elements of the ER and nearby plasmodesmata. Plasmodesmata between mesophyll (above) and bundle sheath cells (below) in control tissues (d) and injected (e) tissues. In (d), note swelling of the central part of the ac of two spatially separated plasmodesmata. In (e), note the electron opaque neck constrictions (arrows) in a plasmodesma showing a clearly defined swelling of the ac. Plasmodesmata connecting bundle sheath (above) and companion cells (below) in control (f) and injected (g) tissues. Arrow (g) indicates apparent continuity between the labeled ER element and the plasmodesmatal neck constriction. Asymmetrically branched plasmodesmata interconnecting sieve elements (above) and companion cell (below) in control (h) and injected (i) tissues. Note the large cavity or pore (c) on the side of the sieve element. Arrows indicate the extent of the ac.

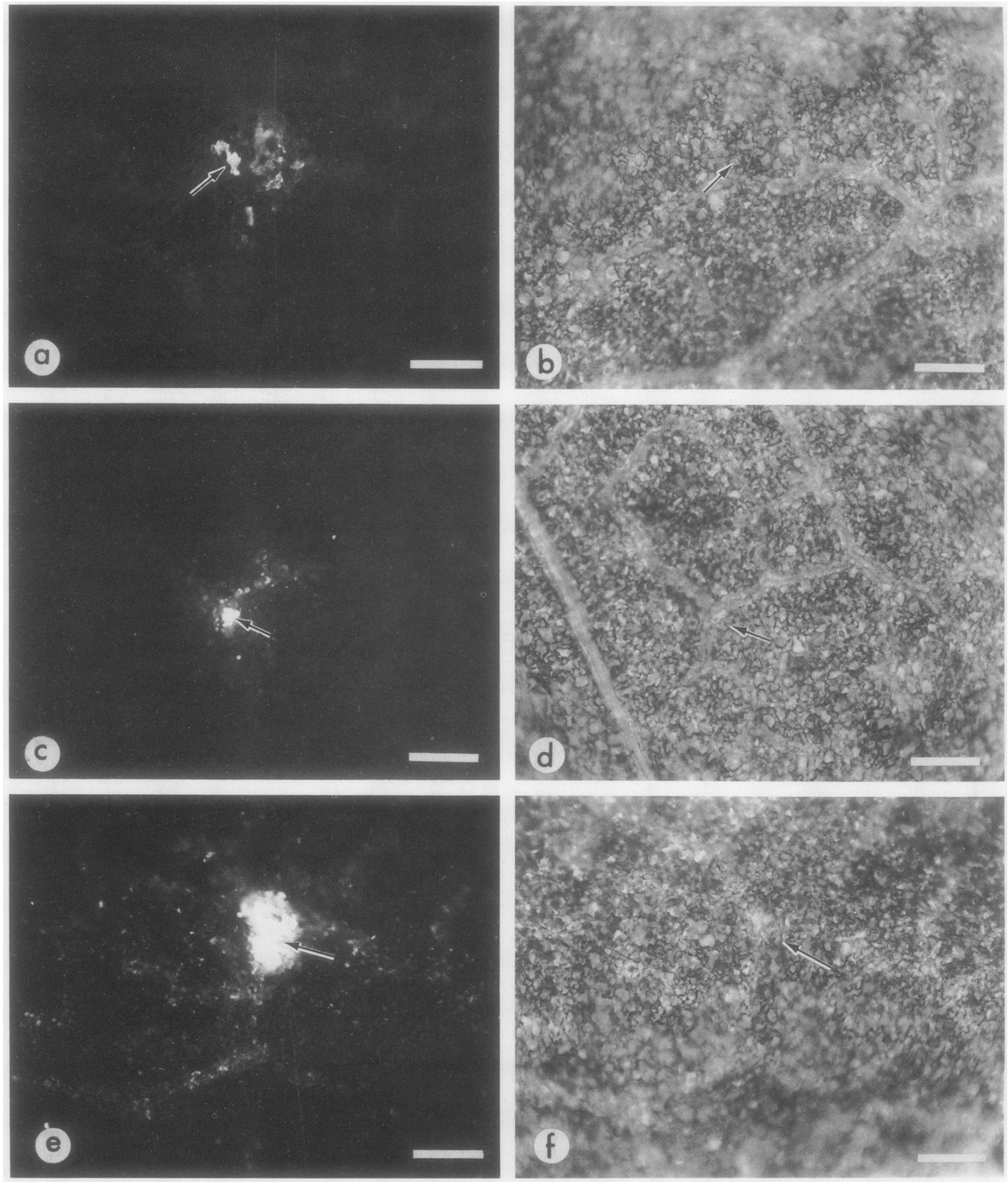

FIG. 6. Effect of PCMBS pretreatment on movement of Lucifer Yellow in the *Ipomea* source leaf symplast. Tissues were equilibrated for 60 min in K-phosphate (pH 7.0) or Mes-KOH buffer (pH 5.5). A 15 min pretreatment (±2 mM PCMBS) was then employed, and PCMBS-free buffer was reapplied. All bars = 200 μm (×64). (a), Control injection site, pretreated in K-phosphate (pH 7.0) before dye injection. (c), PCMBS injection site, pretreated in K-phosphate (pH 7.0) plus 2 mM PCMBS. (e), PCMBS injection site, pretreated with aqueous 2 mM PCMBS, then with 25 mM Mes-KOH buffer (pH 5.5) during and following the injection period. All micrographs were taken 60 min after injection. Bright field micrographs (b), (d), and (f) depict regions illustrated in (a), (c), and (e), respectively.

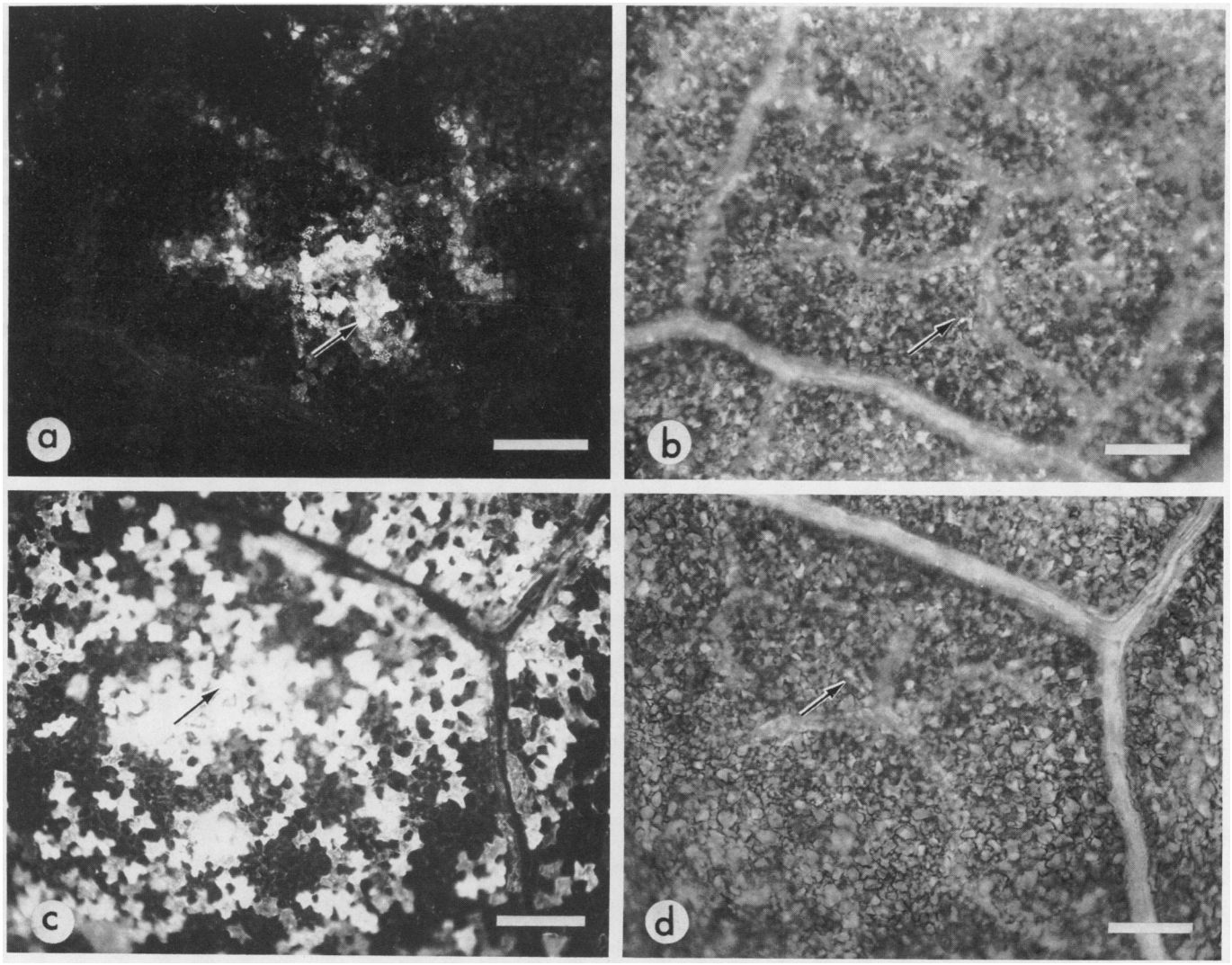

FIG. 7. Effect of bathing medium pH on movement of Lucifer Yellow in the source leaf symplast of *Ipomea*. All bars = 200 μm (×64). Pattern of dye movement 60 min after injection into tissue given a pH 5.5 (a) or pH 8.0 (c) treatment. Bright field micrographs (b) and (d) depict regions illustrated in (a) and (c), respectively.

ies on assimilate transport. The technique described in this paper provides a starting point, opening the door to the cytosol.

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